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Association between intestinal lymphangiectasia and expression of inducible nitric oxide synthase in dogs with lymphoplasmacytic enteritis

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ABSTRACT. Intestinal lymphangiectasia (IL) is a common complication in dogs. Since nitric oxide (NO) is known to relax the lymphatic vessel, we evaluated inducible NO synthase (iNOS) expression using immunohistochemistry in 13 dogs with lymphoplasmacytic enteritis (LPE) with or without IL. The duodenal iNOS expressing cells were significantly increased in dogs with IL-negative or IL-positive LPE dogs (P=0.025, P=0.007) compared with control dogs. However, there was no significant difference in iNOS expression between IL-positive and IL-negative tissues. Based on these results, there is no clear evidence for the NO overproduction in the pathogenesis of IL in dogs with LPE. Factors other than NO could, thus, contribute to IL in dogs with LPE.

KEY WORDS: dog, immunohistochemistry, inducible nitric oxide synthase, intestinal lymphangiectasia, lymphoplasmacytic enteritis

Intestinal lymphangiectasia (IL) is often observed in dogs with chronic small intestinal diseases, including lymphoplasmacytic enteritis (LPE), small- and large-cell gastrointestinal lymphomas, and primary IL [5, 14, 18]. IL sometimes causes lymph leakage into the intestinal lumen, leading to protein-losing enteropathy. Previous histopathological studies showed an association between protein-losing enteropathy and IL in the villus and lamina propria in dogs [26, 27]. Although IL is one of the common complications in dogs with chronic small intestinal disease, its molecular mechanism has not yet been clarified.

Nitric oxide (NO) is a mediator produced from L-arginine by NO synthase [1, 12, 23]. NO synthase has three isoforms: neuronal NO synthase, inducible NO synthase (iNOS), and endothelial NO synthase [1, 6]. In the small intestine, iNOS is expressed in the epithelium and lamina propria [8]. Intestinal NO is known to have an antimicrobial activity or plays an essential role in repairing injured epithelium [3, 8]. In addition to these functions, NO relaxes lymphatic vessel smooth muscle, and the overexpression of iNOS is associated with decreased lymphatic contraction [1, 12, 23]. Indeed, inhibition of iNOS ameliorates the decrease of lymphatic transport due to impaired lymphatic collecting vessel contraction caused by chronic inflammation in obese animals [22]. These findings suggest that iNOS overexpression leads to excess production of NO and decrease of lymphatic transport of the lymphatic vessel, which can dilate the lymphatic vessel.

iNOS is temporarily induced by several mediators such as bacterial lipopolysaccharides [1, 6]. Several inflammatory cytokines such as interleukin-1β, tumor necrosis factor-α, and interferon-γ are also known to increase iNOS expression. These cytokine expressions are known to fluctuate in dogs with LPE [7, 9, 11, 13, 16, 17]. Thus, intestinal iNOS expression can be induced by inflammatory cytokines in dogs with LPE, which may contribute to the pathogenesis of IL.

Based on this background, we hypothesized that excess production of NO contributes to the pathogenesis of IL in dogs. The present study aimed to use immunohistochemistry to evaluate the expression of iNOS and NO production in dogs. Because the NO half-life is extremely short, a few seconds, it is difficult to evaluate produced NO itself. Instead, 3-nitrotyrosine, a product resulting

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from NO and free radical superoxide, is used as a stable marker of NO production [21].

Dogs that underwent both duodenal and ileal endoscopy from February 2019 to July 2020 to investigate chronic (>3 weeks) clinical signs associated with the gastrointestinal disease were included. Corticosteroids treated dogs within two weeks before the study or dogs diagnosed with gastrointestinal lymphoma were excluded. All dogs were fasted more than 12 hr before the endoscopic examination to avoid the effect on the morphology of the lymphatic vessel. Written consent was obtained from the owners of all dogs included in the study. Samples from the small intestine were fixed in 10% neutral buffered formalin and processed for routine histopathological analysis after paraffin embedding. Hematoxylin and eosin-stained sections were used for the diagnosis and evaluation. Histopathological diagnosis was performed according to the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group [25]. The presence of IL (negative or positive) and severity of inflammation (mild or severe) were also evaluated based on the histopathological standards proposed by WSAVA. We regarded IL-positive when the lacteal represents more than 25% of the width of the villous lamina propria, corresponding to mild, moderate, or severe IL in the WSAVA guideline. The severity of inflammation was assessed by the percentage of inflammatory cells occupying lamina propria. In the present study, we classified the severity of inflammation into two levels: Mild, 25–62.5%, corresponding to mild to moderate inflammation; Severe, 62.5–100%, corresponding to moderate to severe inflammation in the WSAVA guideline. The representative images of hematoxylin and eosin staining in dogs recognized as mild and severe inflammation were shown in Supplementary Fig. 1.

The medical records of each dog were reviewed, and the following information was collected: breed, sex, age, body weight, diet, clinical signs, clinicopathological data. The severity of the clinical signs was assessed using the canine inflammatory bowel disease activity index (CIBDAI) and the canine chronic enteropathy clinical activity index (CCECAI) [2, 10]. The CIBDAI was scored using clinical signs, including attitude/activity, appetite, vomiting, stool consistency, stool frequency, and weight loss [10]. The CCECAI is based on the CIBDAI and includes scores for albumin concentration, peripheral edema, ascites, and pruritus [2]. The clinical information of the included cases was summarized in Supplementary Table 1.

Six beagles were used as the healthy controls. All control dogs were castrated males with a median age of 4.9 (range; 4.5–7.3) years and a median body weight of 10.8 (range; 9.8–11.7) kg. The dogs had no clinical signs of gastrointestinal disease and had not received any drugs. The dogs were considered healthy based on physical examination, complete blood count, and plasma biochemistry. The small intestinal tissues were collected as a part of another study [15] approved by the Animal Care Committee of The University of Tokyo (approval no. P17-108).

Formalin-fixed paraffin-embedded tissues were sectioned at 4-µm thickness and used for immunohistochemical analysis. Heat-induced antigen retrieval was performed by autoclaving the samples for 20 min at 121°C in sodium citrate buffer (pH 6.0) for iNOS. Endogenous peroxidase was blocked using 3% H_2O_2 in methanol for 3 min at room temperature. The sections were blocked with 10% goat serum in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 30 min at room temperature and then incubated overnight at 4°C with primary antibodies. Anti-iNOS antibody (rabbit polyclonal, catalog number ab15323, 1:200; Abcam, Cambridge, UK), and anti-3-nitrotyrosine antibody (mouse monoclonal, clone 39B6, catalog number ab61392, 1:1,000; Abcam) were used in this study. After washing with TBS-T, the samples were incubated for 30 min at room temperature with the Envision+ system HRP-labeled polymer (DAKO, Glostrup, Denmark). The color was developed using 3,3-diaminobenzidine for 3 min. Counterstaining was performed with hematoxylin. Positive iNOS staining was confirmed for inflammatory cells infiltrating lamina propria (Supplementary Fig. 2A), which was not observed in negative control slides stained with isotype-matched antibody (Supplementary Fig. 2B). Epithelial cells were also faintly stained for iNOS, as previously described [8]. However, we did not further evaluate iNOS staining in epithelial cells because epithelial cells were uniformly stained in all intestinal samples. Staining of 3-nitrotyrosine was utilized by clone 39B6, which has not been validated in dogs before. Because the NO and superoxide, which produce 3-nitrotyrosine, are overproduced by bacterial infection [19], we used a submucosal abscess specimen as a positive control for 3-nitrotyrosine staining. The anti-3-nitrotyrosine antibody showed diffuse positive staining (Supplementary Fig. 2C), while not by isotype-matched antibodies (Supplementary Fig. 2D).

Five well-oriented lacteals were chosen from each slide using a light microscope with a 20 × objective lens and 10 × eyepiece lens. Digitized images were evaluated using imaging software (ImageJ; National Institute of Mental Health, Bethesda, MD, USA). The number of iNOS-positive cells in the lamina propria was counted, and the results were expressed as iNOS-positive cells per 10,000 µm^2. The 3-nitrotyrosine staining intensity was classified as negative or positive. The sample was recognized as negative when no lacteals were stained. If more than one lacteal was stained, we identified the sample as positive. The immunohistochemical analyses were carried out by a veterinarian who was blinded to the case data.

Statistical analyses were performed using RStudio v.1.1.463 (RStudio, Boston, MA, USA). Since the number of dogs in the present study was too small to analyze the normality of the data, comparisons between 2 groups were made by the Mann-Whitney *U* test and comparisons among 3 groups by the Dunn-Bonferroni test. In addition, Fisher's exact test and Pearson correlation coefficients were also used in the present study. Statistical significance was set at $P<0.05$.

Thirteen dogs were finally included in this study. All dogs were diagnosed with LPE by histopathological examination. The breeds were as follows: Yorkshire Terrier (n=3), Toy Poodle (n=2), mixed breed (n=2), and one each of American Cocker Spaniel, Boston Terrier, French Bulldog, Jack Russell Terrier, Maltese, and Pug. There were eight castrated males, one intact male, three spayed females, and one intact female. The median age was 9.6 (range; 5.8–13.1) years. Clinical signs associated with gastrointestinal disease were as follows: diarrhea (n=9), ascites (n=8), weight loss (n=6), lethargy (n=6), anorexia (n=5), and vomiting (n=3). The median CIBDAI and CCECAI in dogs with LPE were 3.5 (range; 1–11) and 8 (range; 1–14), respectively. The low-fat and ultra-low-fat diets were fed in 6 and 2 of 13 dogs, respectively. Ten and eleven of 13 dogs showed IL in duodenal and ileal samples, respectively. Regarding the severity of inflammation, 10 and 3 duodenal samples showed mild and severe

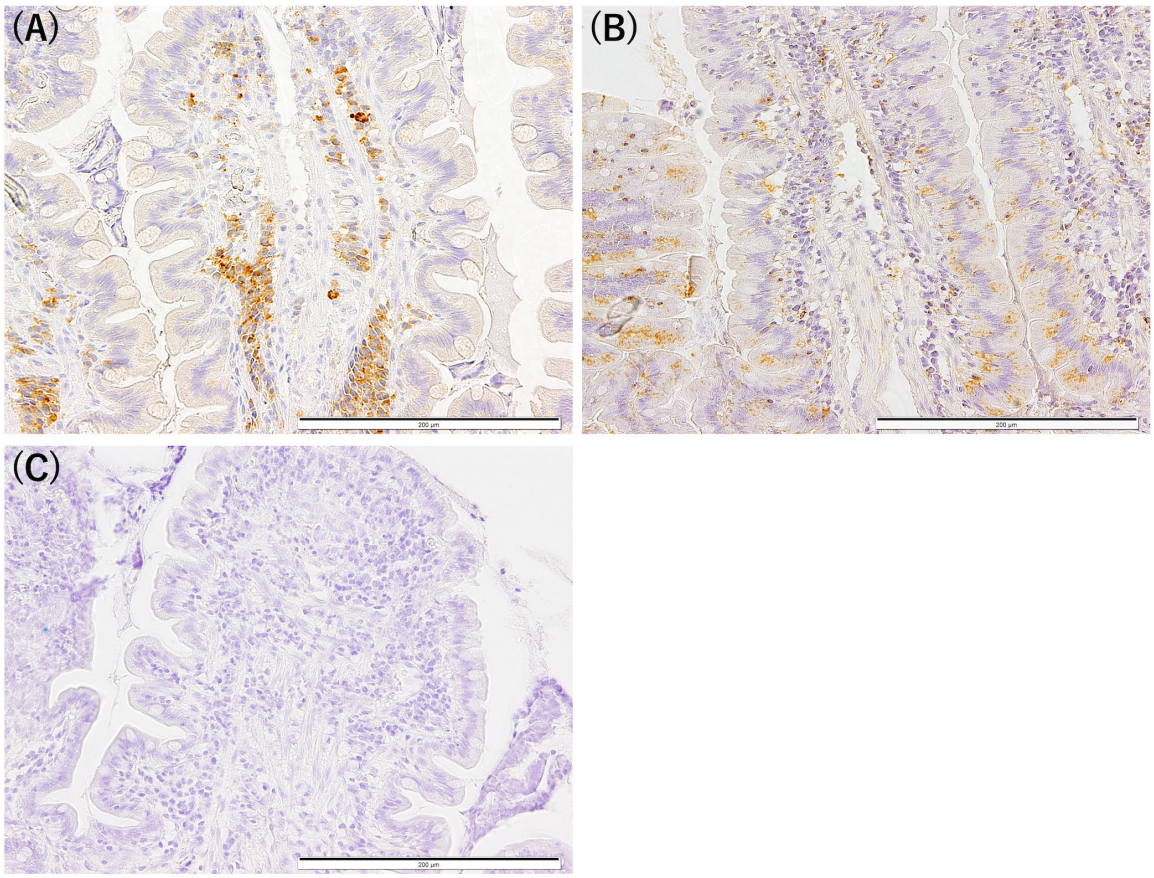

Fig. 1. Representative images of immunohistochemistry for inducible nitric oxide synthase (iNOS) (A) and 3-nitrotyrosine (B and C). The cytoplasm of inflammatory cells infiltrating lamina propria is positively stained for iNOS (**A**). A faint granular expression of 3-nitrotyrosine was observed in epithelial cells and lamina propria and recognized as 3-nitrotyrosine positive (**B**). No staining was observed, and the specimen was recognized as 3-nitrotyrosine-negative (**C**). Bar, 200 µm

inflammation, respectively, while 11 and 2 ileal samples showed mild and severe inflammation, respectively. The median plasma albumin concentration in dogs with LPE was 1.9 (range; 1.1–3.2) g/dl, which was significantly lower than the median value of 3.4 (range; 3.1–3.7) g/dl (P=0.002) in the control dogs. Hypoalbuminemia (<2.6 g/dl) was observed in 10 of 13 dogs.

First, the lamina propria cells positively stained for iNOS were counted (Fig. 1A). The median values of duodenal iNOS-positive cells/10,000 µm^2 in dogs with IL-negative and IL-positive LPE were 15.8 and 14.9, respectively, and significantly higher than the control dogs with median values of 3.1 (Fig. 2A, P=0.025 and P=0.007). In the ileum, there was no significant difference in iNOS-positive cell counts/10,000 µm^2 between control dogs and dogs with IL-negative or IL-positive LPE (Fig. 2B, both P>0.05). Contrary to our hypothesis, there was no significant difference in the number of iNOS-positive cells between dogs with IL-negative and IL-positive LPE in the duodenum and ileum (both P=1.0).

Next, the dogs diagnosed with LPE were divided into two groups based on the severity of inflammation. The median numbers of duodenal and ileal iNOS-positive cells/10,000 µm^2 were 15.4 (range; 6.1–23.7) and 5.2 (range; 0.3–6.8) in dogs with mild inflammation; 10.4 (range; 7.5–16.5) and 3.1 (range; 1.7–4.5) in dogs with severe inflammation. There was no significant difference between the two groups (duodenum, P=0.287; ileum, P=0.769).

We then analyzed the correlation between the number of iNOS-positive cells and clinical severity scores, CIBDAI and CCECAI. There was no significant correlation between the number of iNOS-positive cells and CIBDAI (P=0.597, r=0.170) or CCECAI (P=0.983, r=0.007) in duodenum as well as in ileum: CIBDAI (P=0.201, r= −0.397) and CCECAI (P=0.664, r= −0.140).

Further, the relationship between 3-nitrotyrosine staining and IL was assessed (Table 1). A faint granular expression of 3-nitrotyrosine was observed in epithelia and the lamina propria in positive tissues (Fig. 1B), while no staining was recognized in 3-nitrotyrosine-negative tissues (Fig. 1C). There was no significant difference in the duodenal and ileal 3-nitrotyrosine staining among control dogs and dogs with IL-negative or IL-positive LPE (duodenum, P=0.472; ileum, P=0.137). The median numbers of duodenal and ileal iNOS-positive cells/10,000 µm^2 were 10.4 (range; 1.6–23.7) and 5.4 (range; 0.1–6.8) in dogs with positive 3-nitrotyrosine staining, and 14.2 (range; 0.8–22.3) and 1.7 (range; 0.2–6.8) in dogs with negative 3-nitrotyrosine staining. Differences in iNOS-positive cell counts between 3-nitrotyrosine positive and negative groups were not significant in either duodenum (P=0.287) or ileum (P=0.769).

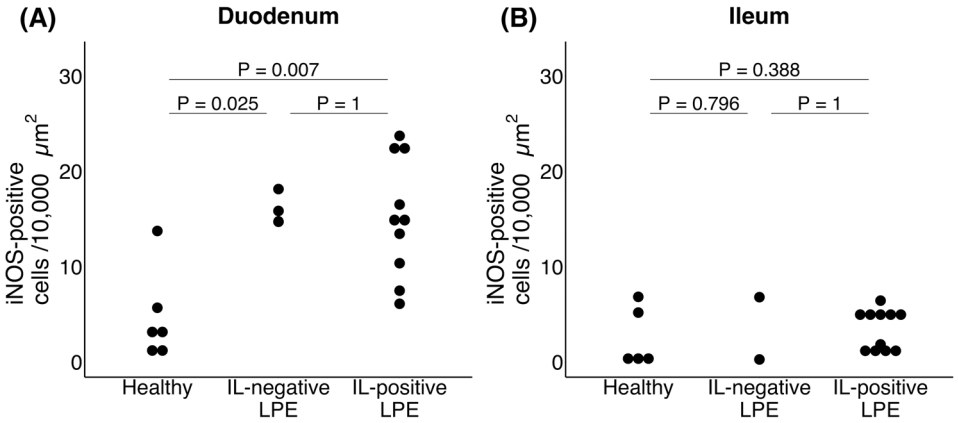

Fig. 2. The numbers of duodenal (**A**) and ileal (**B**) inducible nitric oxide synthase (iNOS)-positive cells/10,000 μm^2 in controls, and intestinal lymphangiectasia (IL)-negative and IL-positive dogs with lymphoplasmacytic enteritis (LPE). Compared with control, the number of duodenal iNOS-positive cells was significantly increased in IL-negative (P=0.025) and IL-positive (P=0.007) dogs with LPE. In contrast, there was no significant difference between IL-negative and IL-positive dogs with LPE (P=1.0). There was no significant difference in the number of ileal iNOS-positive cells among the groups (all P>0.05). Statistical analysis was performed using the Dunn-Bonferroni test.

Table 1. The results of 3-nitrotyrosine staining in control dogs and dogs with intestinal lymphangiectasia negative or positive lymphoplasmacytic enteritis

		Duodenum			Ileum		
		Control	IL-negative LPE	IL-positive LPE	Control	IL-negative LPE	IL-positive LPE
3-nitrotyrosine staining	Negative	3	3	6	3	2	11
	Positive	3	0	4	2	0	0

Data are presented as the number of dogs. IL, intestinal lymphangiectasia; LPE, lymphoplasmacytic enteritis.

Contrary to our hypothesis, there was no significant relationship between IL and the expression of iNOS. Combined with the data that 3-nitrotyrosine was not elevated in IL-positive tissues, the amount of NO produced in intestinal tissue seems insufficient to trigger IL. The results suggest that factors other than NO contribute to IL in dogs with LPE. There are several possible causes of IL in dogs [4]. First, physical obstruction caused by infiltration of inflammatory cells can increase lymphatic pressure, resulting in IL. Second, indirect obstruction due to impaired lymph transport can also cause IL. Although little is known about the mechanism of indirect obstruction, several conditions such as inflammation and obesity result in dysfunction of lymphatic contraction [20, 23, 24]. The dysfunction in lymphatic contraction caused by inflammation and obesity is attributed to the overproduction of NO by iNOS [20, 23, 24]. To our knowledge, little is known about the molecular mechanism that affects the contraction or dilation of lymphatic vessels other than NO. The unknown molecular mechanism for dilation of the lymphatic vessel might be involved in IL in dogs.

A significant increase in duodenal but not ileal iNOS-positive cells in dogs with LPE was observed. On the other hand, there was no significant relationship between the severity of inflammation evaluated by histopathological examination and the number of iNOS-positive cells. Therefore, we assume that the expression of iNOS does not simply attribute to histopathological inflammation. The difference in the dietary antigenicity is one possibility for the discrepancy between the duodenum and ileum. The dietary antigenicity decreases as the foods are digested along the small intestine. Therefore, high antigenicity of the undigested duodenal content compared to well-digested ileal content might be the reason for the discrepancy between the duodenum and ileum. In addition, not all of the inflammatory cells in the small intestine are expressing iNOS. Other possibilities for the discrepancy between duodenum and ileum are differences in the subset of inflammatory cells or inflammatory cytokines. Several inflammatory cytokines such as interleukin-1β, tumor necrosis factor-α, and interferon-γ [1, 6] induce iNOS expression; however, the subset of inflammatory cells or cytokines were not evaluated in the present study. Evaluation of the subset of inflammatory cells by immunohistochemistry and/or quantification of inflammatory cytokines in the duodenum and ileum would be informative to clarify the pathophysiological roles of iNOS in dogs with LPE.

Our study has several limitations. First, the dogs included in the present study mainly had mild-to-moderate LPE based on the median CIBDAI and CCECAI of 3.5 and 8, respectively. In addition, although all the dogs included in the present study were diagnosed with LPE, we could not wholly exclude small-cell lymphoma because of the difficulty in differentiating these diseases. Also, the control dogs' age, breed, and sex did not match dogs diagnosed with LPE because of the limited availability of control dogs. Moreover, the number of dogs with LPE was small, and they had various underlying diseases for LPE; even parasite

infection has not completely been excluded (Supplementary Table 1).

In conclusion, the number of iNOS-positive cells was significantly increased in dogs with LPE; however, there is no clear evidence for NO's involvement in the pathogenesis of IL in dogs with LPE. Factors other than NO contribute to IL in dogs with LPE. Further studies are necessary to clarify the molecular mechanisms of IL in dogs with LPE.

CONFLICTS OF INTEREST. The authors have nothing to disclose.

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